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ISOLATION, IDENTIFICATION OF BROMOPHENOL COMPOUND AND ANTIBACTERIAL ACTIVITY OF KAPPAPHYCUS sp

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ABSTRACT

Extraction, isolation and partial purification of bromophenol compound in *Kappaphycus sp*. was carried out using TLC method. Antibacterial studies for specific bromophenol compound by disc diffusion method were conducted. Identification of a purified component was carried out by NMR technique. FTIRS and mass spectroscopy for elucidation of molecular structure of specific bromophenolic compound were done. From TLC studies, various bands were observed under UV light possessing the maximum value of RF 0.83 corresponding to RF1 band. The band was taken out and tested for antibacterial activity and detection of specific compound. Antimicrobial studies were carried out against various pathogens, *Pseudomonas fluorescence, Staphylococcus aerus*, *Vibrio cholera*, *Proteus mirabilis* and it was observed that the zone of inhibition for specific compound isolated from TLC was very prominent in case of *Pseudomonas fluorescence and Staphylococcus aerus.* C^{13} –NMR spectrum of Bromophenol derivative in $CdCl_3$ showed eight Carbon signals. In mass spectrum analysis of Bromophenol compound, the compound was found to contain two Br atoms from the molecular ion peak at m/z 349.1676 and was analyzed for chemical formula C_{11} H_9 O_3 Br_2. Based on the above characterization the compound was confirmed as-(E)-3-(2, 3-dibromo 4, 5-dihydroxyphenyl)-2-methyl-propenal.

KEYWORDS

Red algae; Kappaphycus, bromophenol, antibacterial

INTRODUCTION

Seaweeds have been widely used for human consumption in many parts of the world. Marine algae can serve as a source of minerals, vitamins, free aminoacids and polyunsaturated fatty acids. Macroalgae can be classified as red algae (*Rhodophyta*), brownalgae (*Phaeophyta*) or green algae (*Chlorophyta*) depending on their nutrient and chemical composition. Red and brown algae are mainly used as human food sources. Seaweed species are rich in beneficial nutrients, in countries such as China, Japan and Korea, they have been commonly utilized in human alimentation. Seaweeds have been consumed in Asia since ancient times. Further, marine algae have been utilized in Japan as raw materials in the manufacture of many seaweed food products, such as jam, cheese, wine, tea, soup and noodles and in the western countries, mainly as a source of polysaccharides for food and pharmaceutical uses[1-4]. In Europe, there is an increasing interest in marine seaweeds as a food, nevertheless, at present there are no European union specific regulations concerning their utilization for human consumption. Ke Li et al.[4] determined various chemical constituents of the red alga *Grateloupia turuturu*. The determination of lipid composition in a given species is essential for further studies on lipid metabolism and on the effect of environmental factors.

Seaweeds or marine macrophytic algae are an assemblage of the members of chloropyceae, phaeophyceae and rhodophyceae and are the common inhabitants of the tidal and intertidal environments of the marine ecosystem. Marine organisms are a rich source of structurally novel and biologically active metabolites. Secondary or primary metabolites produced by these organisms may be potential bioactive compounds of interest in the pharmaceutical industry. Marine algae are exploited mainly for the industrial production of phycocolloids such as agar-agar, alginate and carrageenan, not for health aspects. Seaweeds have some of the valuable medicinal value components such as antibiotics, laxatives, anticoagulants, anti-ulcer products and suspending agents in radiological preparations. Fresh and dry seaweeds are extensively consumed by people especially living in the coastal areas. The edible seaweeds contain a significant amount of the protein, vitamins and minerals essential for the human nutrition. The nutrient composition of seaweed varies and is affected by the species, geographic areas, seasons of the year and temperature of the water. Most of the compounds of marine algae show anti-bacterial activities. Carrageenan polysaccharide derived from algae could be used to inhibit Human Pappilloma Virus (HPV). Marine sources are receiving much attention mainly because of the contents of functional ingredients such as polyunsaturated acids, β-Carotene and their pigments Carotenoids, sulphated polysaccharide (antiviral), and sterols (antimicrobials). Among the different compounds with functional properties, antioxidants are the most widely studied. Moreover the important role of antioxidants in human health has been demonstrated thus increasing the interest in such products and their demand by consumers. Sterols are an important family of lipids, present in the majority of the cells. Because of different routes of synthesis, sterols from plants, fungi and animals show marked differences. The principal sterols in animal and fungal cells are generally cholesterol and ergosterol, respectively. Plant

cells typically contain a mixture of sterols, such as β-sitosterol, stigmasterol and 24-methylenecholesterol.

Marine algae serve as important resources for bioactive natural products [5,6]. Many of these compounds show anti bacterial activities [7], which prompted to investigate the Chinese algae. Brazilian red algae have been found to have phenolic substances. Oxidative stress is an important factor in the genesis of pathology, from cancer to cardiovascular and degenerative disease [8,9]. Marine algae are the excellent source of bioactive compounds such as carotenoids, dietary fibre, protein, essential fatty acids, vitamins and minerals [10-13]. Fayaz et al. [13] suggested the utility of *Kappaphycus alvarezii* for various nutritional products including antioxidant for use as health food or nutraceutical supplement. Sanchez-Machado et al.[12] found that the predominant sterol was desmosterol in red seaweeds (87-93% of total sterol content). Tasende [14] confirmed that fatty acids and sterol of algal class, families and sometimes even species are characteristics to those particular taxa and could be useful as chemotaxonomic.

Choice of solvent for the extraction of antibacterial substances appears to be selective for an alga indicating that the bioactive compounds are not the same for all species. The chemical compounds responsible for the antibacterial activity of marine algae have been identified as organic acids, fattyacids, terpenes, carbonyls,bromophenols, halogenated aliphatic and sulfur containing heterocyclic compounds, isoprenylated and brominated hydroquinones and phlorotannins [15].

Bromophenols have been frequently encountered in various marine organisms including red algae, brown algae, ascidians and sponges, especially, the redalgae of the family rhodomelaceae are known as a rich source of bromophenols which shows pharmacologies activities such as enzyme inhibition, cytotoxic, antioxidant, feeding deterrent, anti-inflammatory and antimicrobial activities [4]. Phenolic compounds have recently received significant attention among various antioxidants and many studies have been performed to identify natural antioxidative phenols with pharmacological activity. Crude extract (odonthalia corymbifera) exhibited moderate antimicrobial activity against various micro organisms. Red algae genus laurencia is an extremely rich source of secondary halogenated metabolites with diverse structural features, particularly of three major classes, sesquiterpenes, diterpenes and acetylenes. Laurencia's halogenated metabolites are known to exhibit biological activity, particularly antibacterial activites. Elatol inhibited 6 sps of bacteria like staphylococcus, klebsiella and salmonella etc. The red algal genus laureneia is unique due to its ability to produce a wide variety of halogenated secondary metabolites with diverse structural features depending on the sps and localities. They have chemical defense substances against marine herbivous is underwable.

In general, from the critical review of literature, it has been observed that the most studies on the nutrient contents of seaweeds have concerned fresh plants. Little is known of the effects of processing by drying or canning. The present investigation aims at on the following from *Kappaphycus sp*:

- Extraction, Isolation and partial purification of bromophenol compound using TLC method
- Antimicrobial test for specific bromophenol compound by disc diffusion method
- Identification of a purified component by NMR

- FTIRS and mass spectroscopy for elucidation of molecular structure of specific bromophenolic compound

MATERIALS AND METHODS

Sample was collected from the sea coast of Rameshwaram, India in the form of dry and living sample. Dried sample was prepared by washing it with water and then with distilled water thoroughly. Then it was kept in sunshade for 7 days. Direct sunlight was strictly avoided because it may cause degradation of active chemical inside the sample. After drying the sample becomes hard enough for grinding. The sample was grinded thoroughly to powder form. Then the powder was used for the estimation of phenol compounds and microbial test. Then again the phenolic compounds were further used for its antimicrobial activity test. Glassware were soaked overnight in cleaning solution and washed thoroughly with running tap water. They were then cleaned with detergent solution and rinsed several times with tap water and finally in distilled water and air dried. The glassware and media were sterilized in an autoclave at 15psi for 20 minutes, at 120^{o}C temperature.

Nutrient Agar per liter/gram (NA):
Peptone-5.0g, Yeastextract-2.0g, NaCl-5.0g Agar-18.0g, Distilled water-1000ml, pH- 7.0

Nutrient broth per litter/gram (NB):
Peptone-5.0g, Yeast extract-2.0g, NaCl-5.0g Distilled water-1000ml, pH- 7.0

Muller Hilton agar:
Beef Extract Powder-2.0 g
Acid Digest of Casein-17.5 g, Starch-1.5 g, Agar-17.0 g, pH- 7.0

Culture collection and maintenance:
The studies of organisms were obtained from CAS in Botany University of Madras, Chennai, India *Staphylococcus aureus, Pseudomonas aeruginosa, Proteus mirabilis, Pseudomonas* sp. The organisms were maintained in Nutrient agar (NA) and also these organisms were maintained at 4^{o}C in slants as a mother.

Extraction and isolation of Bromophenol compound for analysis:
The air dried red algae soaked in Methanol in water 95:5 at room temperature for 6 days. The Methanolic extraction of the red algae was filtered and evaporated under pressure at 400^{o}C. Residue was dissolved in distilled water and then partitioned using Ethyl acetate. The Ethyl acetate was loaded on Silica gel or TLC. The mobile phase was standardized after trying different ratios of Hexane, Chloroform and methanol. The best ratio found out to be 1:0.5:0.1(Hexane: Chloroform: Methanol).After the mobile phase was standardized, the particular ratio was used for TLC. After thin layer chromatography was performed the plates were observed in UV-light and UV-visible light.

Partial purification of compound using TLC:
The crude methanol extract was spotted on to the pre-coated TLC plates (60 F2 54) and developed with a solvent system of hexane, chloroform and methanol in the ratio of 1:0.5:0.1. The developed plate was viewed under UV (260 nm). The spots were eluted with chloroform as solvent and used to study its antibacterarial activity.

Antimicrobial test:
All the bands can be observed only in UV-light. The first band i.e. having RF1 value was marked with a needle, carefully taken out and kept in a test tube. It was dissolved in Ethyl Acetate and centrifuged at 6000rpm for 5minutes.The process was repeated several times and supernatant was collected. The

supernatant was divided in to two parts one is kept for anti-microbial test and another part was kept for NMR to detect specific compound. The antimicrobial assays were done by disc-assay method. The extract was applied over sterile filter paper discs (5mm) in increasing strength i.e. to 1st disc 25µl, to 2nd disc 50 µl , to 3rd disc 75µl, to 4th disc 100µl. After allowing the solvent to evaporate, the discs were placed in nutrient agar test plates inoculated with fresh culture of the test pathogen(106bacteria /ml) in Brain heart infusion broth.

A disc loaded with only solvent was similarly prepared as a control. Five test pathogens were taken they are *Pseudomonas fluorescence, Staphylococcus aerus*, *Vibrio cholera*, *Proteus mirabilis*. The plates were incubated at 37°C. The zone of inhibition of bacteria around the disc was measured and the assay was scored positive (+) if it was <2mm,doubly positive (++) if the zone was =2mm, triple positive (+++) if the zone of inhibition was=7mm and negative (-ve) if there was no inhibition of microbial growth [16].The process described in Bromophenol extraction is repeated up to Ethyl acetate partitioning and the extract is directly used for another antimicrobial test after condensing it to 1ml.

NMR:

The purified compound was identified by two dimensional correlated C^{13} NMR spectra were recorded at 100 MHz on JEOL GsX400 spectrophotometer as indicated, chemical shifts were reported in ppm(δ) using Tetramethylenesilane as internal standard and coupling constants were expressed in Hertz.

Fourier Transform - Infra Red Spectroscopy:

Vibrational spectroscopy is an extremely useful tool in the elucidations of molecular structure. The spectral bands can be assigned to different vibrational modes of the molecule. The various functional groups present in the molecule can be assigned by a comparison of the spectra with characteristic functional group frequencies. As the positions of the bands are directly related to the strength of the chemical bond, a large number of investigations including intermolecular interactions, phase transitions and chemical kinetics can be carried out using this branch of spectroscopy. In IR spectroscopy, the resonance absorption is made possible by the change in dipole moment accompanying the vibrational transition. The Infrared spectrum originates from the vibrational motion of the molecule. The vibrational frequencies are a kind of fingerprint of the compounds. This property is used for characterization of organic, inorganic and biological compounds. The band intensities are proportional to the concentration of the compound and hence qualitative estimations are possible. The IR spectroscopy is carried out by using Fourier transform technique. Infra red spectroscopy involves study of the interaction of electromagnetic radiation with matter. Due to this interaction, electromagnetic radiation characteristic of the interacting system may be absorbed (or emitted). The experimental data consist of the nature (frequency of wave length) and the amount (intensity) of the characteristic radiation absorbed or emitted. These data are correlated with the molecular and electronic structure of the substance and with intra- and inter molecular interactions.

The sample is prepared based on the physical form of the sample to be analyzed. Liquid:CsI / TlBr Cells. Viscous liquids can be smeared in the cell and directly measured. For dilute solutions, liquid cells and variable path length cells are employed. The procedure for recording the %T or %A is as follows:

Air is first scanned for the reference and stored. The sample is then recorded and finally the ratio of the sample and reference data is computed to give required %T or %A at various frequencies. Study of substances with strong

absorbance bands and weak absorbance bands as well as possible. Small amount of samples are sufficient. High resolution is obtained.

Procedure:

Preparation of samples for infrared measurements and infrared spectra typically, 1.5 mg of compound, was dissolved in the chloroform. The concentrated compound was placed in CaF2 windows and a 6 Am tin spacer or a 25 Am Teflon spacer for the experiments in H_2O or $2H_2O$, respectively. FT-IR spectra were recorded by means of a Perkin-Elmer Spectrum-1 FT-IR spectrometer using a Deuterated Triglycine sulfate detector. At least 24 h before, and during data acquisition, the spectrometer were continuously purged with dry air at a dew point of 40 jC. Spectra of sample were acquired at 2 cm-1 resolution under the same scanning and temperature conditions. In the thermal denaturation experiments, the temperature was raised in 5 jC steps from 20 to 95 jC. Before spectrum acquisition, samples were maintained at the desired temperature for the time necessary for the stabilization of temperature inside the cell (6 min). Spectra were collected and processed using the SPECTRUM software from Perkin-Elmer. Correct subtraction of H_2O was judged to yield an approximately flat baseline at 1900–1400cm^{-1}, and subtraction of $2H_2O$ was adjusted to the removal of the $2H_2O$ bending absorption close to 1220 cm^{-1} .

OBSERVATION AND RESULT

Various bands under UV light on TLC plate can be seen in Figs. 1 and 2. Table 1 shows various RF value for different band on TLC plate under UV light. Table-2 shows the inhibition of microorganisms by specific bromophenol compound. The zone of inhibition for specific compound (By Bromophenol extraction)isolated from TLC was very prominent in case of *Pseudomonas fluorescence (*Fig. 3) *and Staphylococcus aerus* (Fig. 4) and moderate in case of *Vibrio cholera* (Fig. 5) and zone of inhibition were minimum in *Proteus mirabilis* (Fig. 6) which shows good antimicrobial activity. From the qualitative analysis of bromophenol compound, it is concluded from the antimicrobial test that the compound having RF value 0.83 which is a specific Bromophenol compound has got significant anti-microbial activity against many pathogens. C^{13} –Nuclear Magnetic Resonance **(**Fig. 7) spectrum of bromophenol derivative in $CdCl_3$ showed eight carbon signals. A peak at 178.3 due to keto carbonyl and aromatic carbons appeared between 130.0-129.7. Infra-Red spectroscopy (Fig. 8) shows peak at 3428 cm^{-1} due to –OH stretching frequency, 1744 cm^{-1} due to keto carbonyl, 1633 cm^{-1} due to alkene group and 743 cm^{-1} due to C-Br stretching frequency. Based on the above results structure was confirmed as bromophenol derivative. In mass spectroscopy **(**Fig. 9**)** analysis, the compound was found to contain two Br atoms from the molecular ion peak at m/z 349.1676 and was analyzed for C_{11} H_9 O_3 Br_2. Based on the above characterization the compound was confirmed as (E)-3-(2, 3-dibromo 4, 5-dihydroxyphenyl)-2-methyl-propenal (Fig.10).

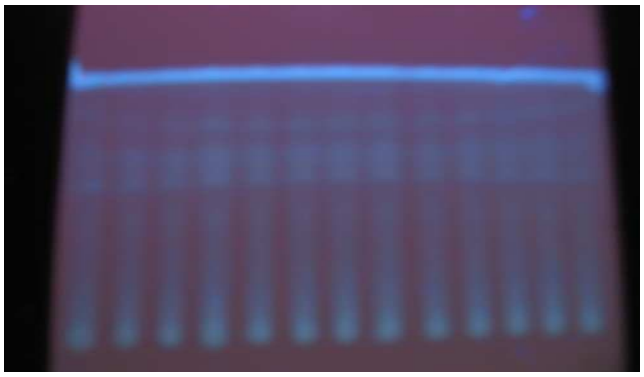

Figure 1
Bands under UV light on TLC plate

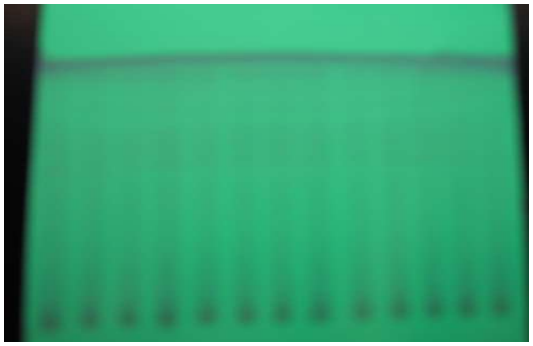

Figure 2
Bands under UV-visible light on plate

Table 1
RF value for different band on TLC plate under UV light

SI. NO.	BAND	RF VALUE
1.	RF1	0.83
2.	RF2	0.76
3.	RF3	0.72
4.	RF4	0.66
5.	RF5	0.59

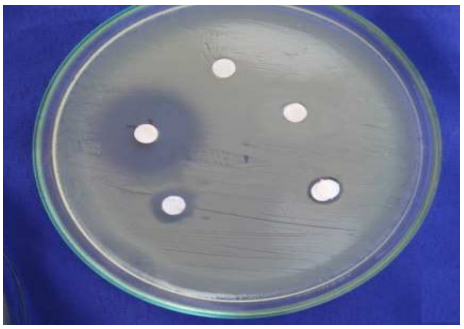

Figure 3
Plate-1. Inhibition of Pseudomonasfluorescence

Figure 4
Plate-2. Inhibition of Staphylococcus aerus

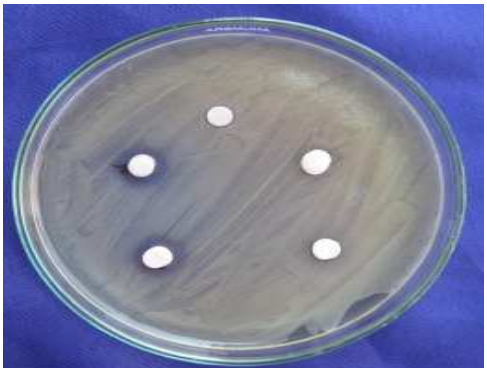

Figure 5
Plate- 3. Inhibition of Vibrio Cholera

Figure 6
Plate-4. Proteus mirabilis

Table2
Inhibition of Microorganisms by Specific Bromophenol Compounds

Vol. (in µl)	Plate-1 *Pseudo-monas fluore-scence*	Plate-2 *Staphylo-coccus aerus*	Plate-3 *Vibrio cholera*	Plate-4 *Proteus mirabilis*
25	-	+	-	-
50	+	++	-	-
75	++	+++	+	-
100	+++	+++	++	+

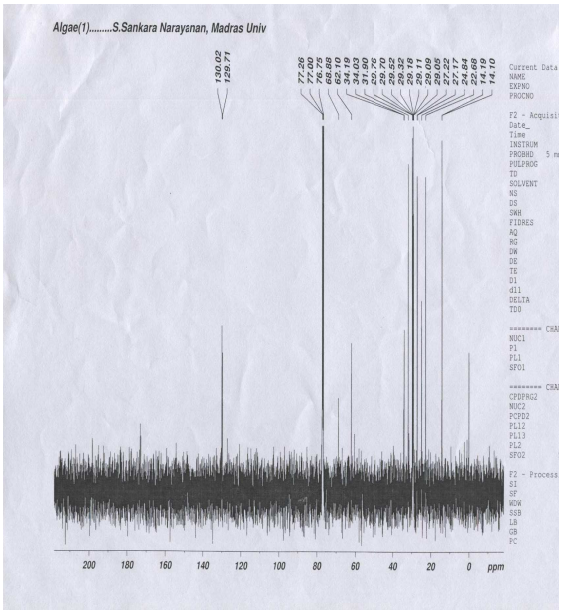

Figure 7
C^{13}-NMR for Bromophenol Compounds

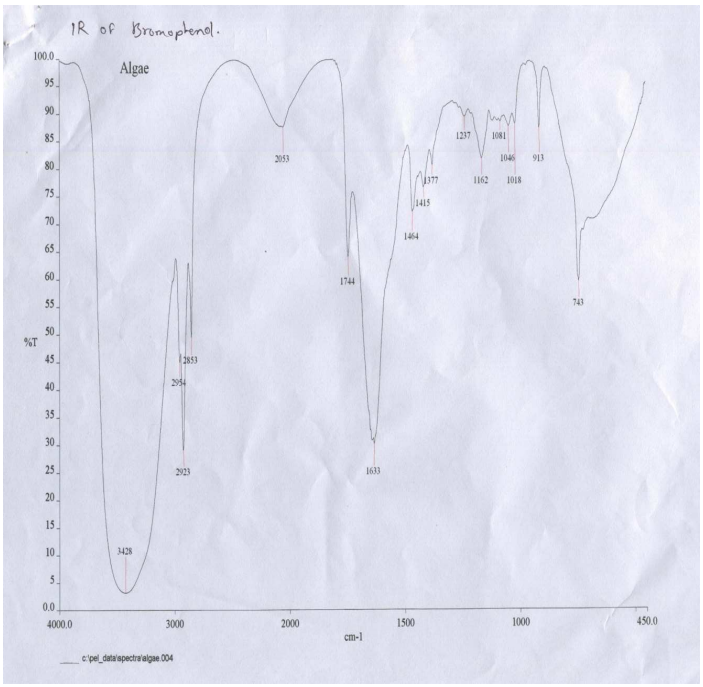

Figure 8
IR- for Bromophenol Compounds

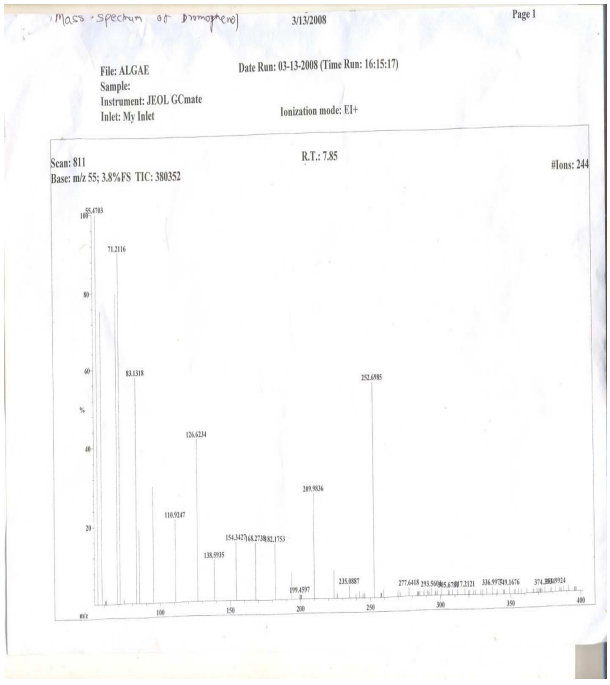

Figure 9
Mass Spectrum of Bromophenol Compound

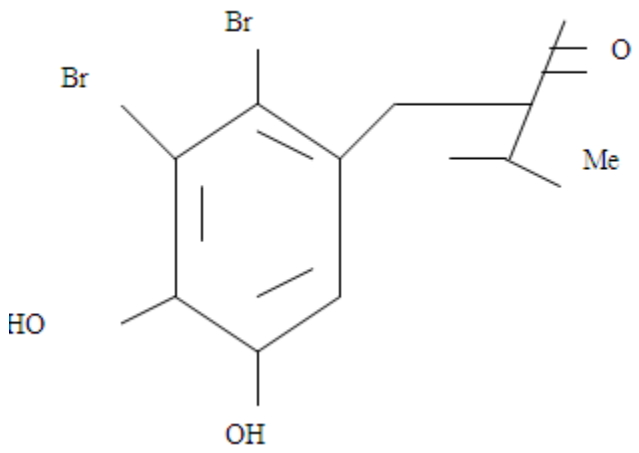

Figure10
Structure of (E)-3-(2, 3-dibromo 4, 5-dihydroxyphenyl)-2-methyl-propenal

Dhamotharan [17] reported that brown algae have been shown to inhibit the growth of fungal pathogens, namely, *microsporuncanis, candidaalbicans, aspergillusfumigatue*. The extract residue of stoecho showed max activity against aspergillus funigatus only. But the same showed padina- highly active against aspergillu funigatus compared with other test organisms (candia, Microsporum, epidermophyton). The observations showed trhe solvent extract residue of Padina to be effective against aspergillus and microspurum whitle that stoc to be effective against aspergillus only. The two exp. algae recorded maxi activity against staphylococcus aureus-bacillus, klebsiells, shigella and vibrio also inhibited. The extract residues of both algae did not show any effect on the growth of proteus vulgaris.similarly,padina-not shown against pseudomonas. Inci Tuney et al. [18] tested the methanol, acetone, diethyl ether and ethanol extracts of 11 seaweed species in vitro for their antimicrobial activities against Candida sp., Enterococcus, Staphylococcus aureus, Streptococcus epidermidis, Pseudomonas aeruginosa and Escherichia coil with the disc diffusion method. It was found that diethyl ether was the best solution for extracting the effective antimicrobial materials from the algae species. A significant difference in antimicrobial activity was not observed between acetone and methanol extracts of each alga. Vairappan et al. [7] isolated two halogenated C_{15} acetogenins, named lembyne-A and lembyne-B from an unrecorded *Laurencia* species collected off the Malaysian waters. Their structures were deduced on the basis of spectroscopic evidence. Elatol and iso-obtusol showed potent antibacterial activity against some marine bacteria. Vairappan et al. [19] investigated the chemical composition of five species of the red algal genus *Laurencia* from coastal waters of Okinawa Prefecture, Japan. The structures of these halogenated metabolites as well as their antibacterial activity against some marina bacteria were reported. Vairappan [20] investigated delves upon extraction, isolation, structural elucidation and antibacterial activity of inherently available secondary metabolites of *Laurencia majuscule* Harvey collected from two locations in waters of Sabah, Malaysia. Two major halogenated compounds, identified as

elatol and isoobtusol were isolated. Elatol inhibited six species of bacteria with significant antibacterial activities against Staphylococcus epidermis, Klebsiella pneumonia and Salmonella and Salmonella sp. Elatol showed equal and better antibacterial activity compared with tested commercial antibiotics while iso-obtusol only equaled the potency of commercial antibiotics against *K. pneumonia* and *Salmonella sp.* Ke Li et al. [21] isolated and identified three new natural occurring bromophenols from the marine red alga *Polysiphonia urceolata*. The structures of these compounds were elucidated by extensive analysis of 1D and 2D NMR and IR spectra and MS data. Hyi-Seung Lee et al. [22] mentioned that bromophenols isolated from the red alga *Odonthalia corymbifera* exhibited potent ICL inhibitory activity and blocked appressoria fromation by *M. grisea* in a concentration dependent manner. The protective effect of bromophenols and their strong inhibition of appressorium formation on rice plants suggest that ICL inhibitors may be promising candidates for crop protection, particularly to protect rice plants against *M. grisea.* Pedersen et al. [23] analysed 23 species of red algae by using TLC and GLC-MS techniques to find simple bromophenols. Ten bromophenols were detected, five of which might be artifacts. Bromophenols were detected in species from the families ceramiaceae, Delessecriaceae, Bonnemaisoniaceae, Rhodophyllaceae, Corallinaceae and Rhodomelaceae.

SUMMARY AND CONCLUSION

Extraction, isolation and partial purification of bromophenol compound and its antibacterial activity in *Kappaphycus sp*. was carried out. Sample was collected from the sea coast of Rameshwaram, Tamil Nadu, India in the form of dry and living sample. From TLC studies, various bands were observed under UV light possessing the maximum value of RF 0.83 corresponding to RF1 band. The band was taken out and tested for antibacterial activity and detection of specific compound. Antimicrobial studies were carried out against various pathogens, *Pseudomonas fluorescence, Staphylococcus aerus*, *Vibrio cholera*, *Proteus mirabilis* and it was observed that the zone of inhibition for specific compound isolated from TLC was very prominent in case of *Pseudomonas fluorescence and Staphylococcus aerus.* C^{13} –NMR spectrum of Bromophenol derivative in $CdCl_3$ showed eight Carbon signals. In mass spectrum analysis of Bromophenol compound, the compound was found to contain two Br atoms from the molecular ion peak at m/z 349.1676 and was analyzed for chemical formula C_{11} H_9 O_3 Br_2. Based on the above characterization the compound was confirmed as (E)-3-(2, 3-dibromo 4, 5-dihydroxyphenyl)-2-methyl-propenal.

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